Cellular Immunology 256 (2009) 86–91

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Depletion of the programmed death-1 receptor completely reverses established clonal anergy in CD4$^+$ T lymphocytes via an interleukin-2-dependent mechanism

Kenneth D. Bishop [a,1], John E. Harris [a,b,2], John P. Mordes [b], Dale L. Greiner [b,c], Aldo A. Rossini [a,b,c], Michael P. Czech [a], Nancy E. Phillips [b,*]

[a] Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA 01655, USA
[b] Department of Medicine, University of Massachusetts Medical School, 373 Plantation Street, Biotech 2, Suite 218, Worcester, MA 01605, USA
[c] Program in Immunology and Virology, University of Massachusetts Medical School, Worcester, MA 01655, USA

ARTICLE INFO

Article history:
Received 12 September 2008
Accepted 22 January 2009
Available online 23 February 2009

Keywords:
T cell
Tolerance
Anergy
PD-1

ABSTRACT

Recent studies have implicated the cell surface receptor Programmed Death-1 (PD-1) in numerous models of T cell anergy, though the specific mechanisms by which the PD-1 signal maintains tolerance is not clear. We demonstrate that the depletion of PD-1 with siRNA results in a complete reversal of clonal anergy in the A.E7 T cell model, suggesting that the mechanism by which PD-1 maintains the anergic phenotype is a T-cell-intrinsic phenomenon, and not one dependent on other cell populations in vivo. We have also shown that the neutralization of IL-2 during restimulation abrogates the effect of PD-1 depletion, suggesting that tolerance mediated by PD-1 is wholly IL-2 dependent, and likewise intrinsic to the tolerized cells.

1. Introduction

The immunosuppressive agents used in current transplantation protocols are known to increase the risk of infection and neoplasia due to their nonspecific dampening of the immune response [1–3]. One alternative to generalized suppression of the immune system is a more tailored approach that seeks to induce a state of selective peripheral tolerance specifically to transplanted grafts [4–7]. Inducing an allo-specific tolerant state would permit the introduction of an organ graft into an otherwise fully competent immune environment, capable of immune surveillance and pathogen eradication. The mechanisms that generate peripheral transplantation tolerance are not yet fully elucidated, but it is known that grafts are rejected as the result of both acute and chronic immune activation [8], processes that involve numerous immune mechanisms [9–11]. It is well accepted that CD4+ T lymphocytes are central to the rejection of allografts and that they are also necessary for the successful induction of tolerance [4,12]. A number of immune processes discovered in animal models that are posited to result in immunological tolerance include clonal deletion, suppression of reactive lymphocyte subsets by regulatory T cells, and T cell anergy [13,14].

CD4$^+$ T lymphocytes require two signals for optimal activation and production of IL-2, which drive entry into the cell cycle and subsequent clonal expansion [15,16]. Signal 1 is delivered through the TCR upon encounter with antigen. When signal 1 is delivered in the absence of a costimulatory signal, known as signal 2, the levels of IL-2 produced are not sufficient to drive clonal expansion. Instead, the T cell acquires a phenotype characterized by antigen unresponsiveness defined as clonal anergy [17]. After a T cell is rendered anergic, it is unable to produce IL-2 or proliferate, even when provided a signal through the TCR in the presence of costimulation. Early studies demonstrated that anergy is an active phenotype that *in vitro* requires protein synthesis and can be prevented by treatment with cycloheximide and cyclosporine A [18]. These findings suggest that anergy is established through a TCR-dependent signal transduction pathway.

The search for factors that participate in this putative anergy pathway is ongoing. A number of genes that are upregulated early in the course of anergy induction in T cells have been identified. These include the transcription factor Egr-2 [19,20] and the E3-ubiquitin ligases Cbl-b [21,22] and GRAIL [23,24]. Recent studies have shown that the products of these genes are each necessary for establishing the anergic phenotype. We have previously shown that Egr-2 is necessary for the induction of anergy, but does not

* Corresponding author. Fax: +1 508 856 4093.
E-mail address: nancy.phillips@umassmed.edu (N.E. Phillips).
[1] Present address: Department of Internal Medicine, Brown Medical School, Providence, RI, USA.
[2] Present address: Department of Dermatology, Hospital of the University of Pennsylvania, Philadelphia, PA 19104, USA.

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doi:10.1016/j.cellimm.2009.01.008

appear to have a role in maintaining unresponsiveness once the anergic phenotype is established [19].

The aims of this study were to identify genes that are differentially expressed during the maintenance phase of anergy and to determine whether they contribute to the anergic phenotype. We show that PD-1, a known negative costimulatory receptor [25], is upregulated in anergic cells for at least five days after anergy induction, and that depletion of PD-1 protein levels with RNAi at this time results in complete, IL-2-dependent reversal of the anergic phenotype. We further show that, at this late time point, the effect of PD-1 depletion is specific to anergic cells, as treatment of fully costimulated cells with siRNA directed against PD-1 does not increase antigen responsiveness.

2. Methods

2.1. Mice

B10.BR ($H2^k$) and BALB/c ($H2^d$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were certified to be free of Sendai virus, pneumonia virus of mice, murine hepatitis virus, minute virus of mice, ectromelia, LDH elevating virus, mouse poliovirus, Reo-3 virus, mouse adenovirus, lymphocytic choriomeningitis virus, polyoma, Mycoplasma pulmonis, and Encephalitozoon cuniculi. They were housed in a specific pathogen free facility in microisolator cages, and given autoclaved food and acidified water *ad libitum*. All animal use was in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical School and recommendations in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996).

2.2. In vitro T cell line

The pigeon cytochrome c (PCC)-specific, and I-E^k restricted $CD4^+$ murine helper T cell clone A.E7 was the gift of R. Schwartz (NIH, Bethesda, MD), and the cultivation of these cells has been described [18]. Briefly, A.E7 T cells were stimulated with irradiated B10.BR ($H2^k$) splenocytes and 8 μM whole PCC (Sigma–Aldrich, St. Louis, MO) for 48 h, expanded 20:1 in 15 U/ml of recombinant IL-2, and cultured for a minimum of 12 days before use in all experiments.

2.3. Anergy induction

A.E7 T cells were isolated over a Ficoll gradient (Lympholyte M, Accurate Chemical & Scientific Corp., Westbury, NY) and anergy was induced by overnight incubation of $20–40 \times 10^6$ cells in a T75 or 100×10^6 cells in a T175 tissue culture flask (BD Falcon, Bedford, MA) that had previously been coated with anti-CD3 monoclonal antibody (clone 145-2C11, BD Biosciences Pharmingen, San Diego, CA) at a concentration of 1 μg/ml. Fully stimulated cell cultures were supplemented with soluble anti-CD28 monoclonal antibody (clone 37.51, BD Biosciences Pharmingen) at a concentration of 1 μg/ml. Mock stimulated cultures received no stimulatory antibodies (untreated). After 12 h, cells were removed from the stimulatory medium, washed, and cultured in fresh medium for varying times as indicated.

2.4. Proliferation assay

Proliferation was assayed by [^{3}H]-thymidine incorporation into DNA. Briefly, cells were cultured with irradiated syngeneic (B10.BR) splenocytes plus increasing doses of PCC antigen for 64–70 h and pulsed during the final 16 h with 1 μCi of [^{3}H]-thymidine.

2.5. Microarray procedures

Microarray analysis on mock-stimulated, anergized, or fully activated A.E7 T cells has been described [19].

2.6. Quantitative real time PCR

Total RNA was prepared using an RNeasy kit (Qiagen). cDNA was synthesized using oligo-dT primers and reverse transcriptase AMV (Roche, Indianapolis, IN) according to manufacturer's instructions. Quantitative real-time PCR was performed in a Light Cycler (Roche) using a LightCycle DNA Master SYBR Green I PCR kit (Roche) and TaqStart Antibody (Clonetech, Mountain View, CA). Primers used were: PD-1: sense: 5′-CCGCTTCCAGATCATACAG-3′, antisense: 5′-CTCTGGCCTCTGACATACTTG-3′, product = 329 bp; and HPRT: sense: 5′-TTAGCGATGATGAACCAGGTTAT-3′, antisense: 5′-TGGCCTGTATCCAACACTTC-3′, product = 490 bp.

2.7. Western blotting

Cells were washed once in cold PBS, lysed in SDS gel-loading buffer without 2-mercaptoethanol (2-ME) or bromophenol blue, boiled for 5 min, and quantified by BCA protein assay (Pierce, Rockford, IL). 2-ME and bromophenol blue were then added to the lysates and 20 μg of protein was run on a 10% SDS gel. Protein was transferred to a PVDF membrane and blots were probed with anti-PD-1 antibody (R&D Systems, Minneapolis, MN) (0.1 μg/ml) followed by anti-goat secondary antibody conjugated to horseradish peroxidase (Chemicon Int., Temecula, CA) diluted 1:50,000 and developed for visualization by enhanced chemiluminescence (Pierce, Rockford, IL). Blots were probed for actin with anti-actin antibody (Sigma–Aldrich)(1:50,000), goat anti-mouse secondary antibody (1:10,000 dilution, Chemicon Int.), and developed by enhanced chemiluminescence (Perkin–Elmer, Boston, MA).

2.8. siRNA transfection

SMARTpool siRNA duplex cocktails were purchased from Dharmacon, Inc. (Lafayette, CO) corresponding to the GenBank nucleotide sequences for Pdcd1 (PD-1) (NM_008798) and Egr-2 (NM_010118). The PD-1 SMARTpool was composed of the siRNA duplexes (sense strand): duplex 1: uau cau gag ugc ccu agu guu; duplex 2: gau gcc cgc uuc cag auc auu; duplex 3: gaa cug gaa ccg ccu gag uuu; duplex 4: gca agg acg aca cuc uga auu. An siRNA duplex with the sequence cag ucg cgu uug cga cug gdt dt that does not correspond to any sequence published in GenBank was used as a control in each of the experiments. The Egr-2 siRNA SMARTpool sequences have been previously published [19].

A.E7 T cells were electroporated with 20 nm of siRNA duplexes in 0.5 ml complete growth medium at a density of 60×10^6 ml^{-1} in a 0.4 cm GenePulser cuvette (BioRad, Hercules, CA). The electroporation was performed with a GenePulser electroporator II (BioRad) at 310 mV, 950 mF. Cells were allowed to recover for 10 min on ice, then added to complete growth medium.

2.9. IL-2 neutralization

To block autocrine IL-2 signaling, 10 μg/ml of IL-2 neutralizing antibody (Clone S4B6, BD Biosciences Pharmingen) was included in the challenge culture. As a control, an equal concentration of rat IgG2a isotype control antibody was used. Proliferation was assessed as described above.